Materials and Methods

Laser Doppler Measurement of Blood Flow from Wildtype and Trpv1$^{-/-}$ Mice

Wild-type (WT) and TRPV1-deficient (*Trpv1*$^{-/-}$) C57B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were bred and genotyped at Model Animal Research Center of Nanjing University, China. Adult male mice (aged >15 weeks, weight ~25 g) were used. For blood flow measurements, mice were anesthetized using Nembutal (80 mg/kg) given by I.P. injection 30 min before experiments. Nicotinic acid (130 mM, dissolved in physiological saline, pH 7.4) or vehicle (physiological saline with osmolarity adjusted to the same level as nicotinic acid solution using glucose, pH 7.4) was administered subcutaneously to the abdomen at a dosage of 120 mg/kg. The change in the ear blood flow was measured using a laser Doppler flowmeter (LDF100C, BIOPAC Systems, Inc.) with fiber-optic based probe (TSD146, BIOPAC Systems, Inc.), an approach similar to that taken in previous studies of both human and murine niacin responses[1-3]. The probe was placed against the dorsal side of the right ear of anesthetized mice to measure the blood flow at 5 min intervals before and after exposure to nicotinic acid or vehicle. Baseline blood flow was established by an average of measurements over 10 min prior to injection of drug or vehicle. Data were analyzed as the percentage change over the baseline blood flow in tissue perfusion units [$(TPU/TPU_{baseline})$ %)].

cDNA Constructs and Cell Transfection

The constructs used in the present study were mouse TRPV1-4 and human TRPV1 cDNAs inserted into the mammalian expression vector pEYFP-N3 [4], which provided an enhanced yellow fluorescence protein (eYFP) tag fused to the C-terminal end of each channel. tsA201 cells were cultured in a DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, at 37°C with 5% CO_2. Cells were passaged 18-24 h before transfection by plating onto glass coverslips coated with 0.1 mg/ml poly-D-lysine to improve cell adhesion and subsequent patch or pH imaging recordings. Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions 24-to-48 h before recording.

Electrophysiological Recordings

Macroscopic and single-channel currents were recorded from channel-expressing cells using a HEKA EPC10 amplifier with PatchMaster software (HEKA). Unless stated otherwise, the recordings were done at room temperature using mouse TRPV1 (mTRPV1) with inside-out configuration. Patch pipettes were pulled from thin-wall borosilicate glass and fire-polished to a resistance of ~2 MΩ. For whole-cell recordings pipettes were pulled from thick-wall borosilicate glass to 3-5 MΩ. Membrane potential was held at 0 mV and, except for testing voltage-dependence of TRPV1 activation, currents were elicited by a 300-ms step to +80 mV followed by a 200-ms step to -80 mV at 1-s intervals. Data were filtered at 2.25 kHz and sampled at 12.5 kHz. For whole-cell recordings the capacity current was minimized by amplifier circuitry, and the series resistance was compensated by 65-80%. Standard symmetrical bath and pipette solutions

contained 140 mM NaCl, 0.2 mM EGTA, 10 mM Glucose and 15 mM HEPES (pH 7.2). When desensitization was studied, 2 mM $CaCl_2$ was added whereas EGTA was removed from extracellular solution. Solution switching was achieved with a rapid solution changer RSC-200 (Biological Science Instruments).

Temperature Control and Monitoring

Temperature control and monitoring were achieved with the same approach as previously reported [5]. Briefly, the perfusion solution was heated using an SHM-828 eight-line heater driven by a CL-100 temperature controller (Harvard Apparatus). To obtain a complete temperature-current relationship, in some experiments the solution was first cooled by embedding the perfusion solution reservoir in ice water. A TA-29 miniature bead thermistor (Harvard Apparatus) was placed about 1 mm from the pipette tip to monitor local temperature change. Temperature readout from the thermistor was fed into an analog input port of the HEKA patch-clamp amplifier and recorded simultaneously with channel current. When experimental temperature was not controlled, recordings were conducted at room temperature at ~24°C.

Fluorescence Imaging of Intracellular pH

TRPV1-expressing tsA201 cells grown on poly-D-lysine-coated 25 mm glass coverslips were washed twice with HEPES-buffered extracellular solution (ECS) that contained (in mM) 140 NaCl, 5 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 10 D-glucose, and 15 HEPES (pH 7.4, ~300 mOsm). Cells were initially incubated in ECS containing 5 μM BCECF-AM (Molecular Probes) for 15 min at 37°C and then placed in a closed perfusion-imaging chamber (Warner) mounted on the stage of a Zeiss Axiovert inverted microscope. Before intracellular pH measurement, cells were perfused with ECS at a constant rate of 3 ml/min for >10 min to wash out extracellular BCECF-AM and to allow intracellular hydrolysis process of the AM ester to complete, which converts the non-fluorescent BCECF-AM into the fluorescent BCECF. After measurement of the baseline level of intracellular pH (pH_i), cells were subjected to ECS supplemented with different concentration of nicotinic acid (pH 7.4) until an equilibrium state was reached at each concentration. At the end of each experiment, the high-K^+-nigericin technique [6] was used to calibrate the ratio of fluorescence emissions to the pH level, with a high-K^+ medium (pH 7.0) containing (in mM) 135 KCl, 0.6 $CaCl_2$, 1.0 $MgCl_2$, 10.1 KOH, 5.1 D-glucose, 20 HEPES, and 3.2 NMDG-Cl, supplemented with 10 μM nigericin. pH_i was calculated from the intensity ratio of fluorescence lights emitted at 535 nm upon excitation at 490 nm and 440 nm respectively (F_{490}/F_{440}). Images were collected every 10 s using an ICCD camera system (Stanford Photonics) and analyzed with the OpenLab image processing software. For each experiment, 20-30 cells were monitored per coverslip. All experiments were performed at room temperature.

Data Analysis

The dose-response relationship was quantified from macroscopic currents and fitted with the Hill equation:

$$\frac{I-I_{min}}{I_{max}} = \frac{[x]^n}{EC_{50}^n+[x]^n} \quad (1)$$

Where I and I_{max} are the steady-state currents in the presence of an agonist at concentration [x] and saturating concentration, respectively, I_{min} is the leak current or, in the case of dual-agonist experiments, the total current elicited in the presence of the first agonist, EC_{50} is the agonist concentration at which activation is half-maximal, and n is the Hill coefficient.

G-V curves were fitted to a single-Boltzmann function:

$$\frac{G}{G_{max}} = \frac{1}{1+e^{(V_{half}-V)/k}} \quad (2)$$

Where G/G_{max} is the normalized conductance, V_{half} is the half-activation voltage, k is the slope factor defined as RT/qF, where q is the equivalent gating charge, T is temperature, R is the gas constant, and F is the Faraday's constant.

Activation threshold temperature was determined from the raising phase of the current-temperature relationship recorded from cells expressing TRPV1 channels [7]. The raising phase exhibited two temperature-dependent phases, a less temperature-dependent phase at lower temperatures (reflecting heat-dependent increase in background current) followed by a higher temperature-dependent phase at higher temperatures (reflecting heat-dependent activation of TRPV1). Each phase was fitted to a linear function. The temperature at the intersection of the two lines was defined as the activation threshold temperature ($T_{takeoff}$).

All values are given as mean ± SEM for the number of measurements (n). Statistical significance was determined using the Student's t test or mixed-model ANOVA.

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